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# Investigation of interactions of a resorcin[4]arene receptor with bilayer lipid membranes (BLMs) for the electrochemical biosensing of mixtures of dopamine and ephedrine

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## Abstract

The present article investigates the interactions of a resorcin[4]arene receptor with planar bilayer lipid membranes (BLMs) that can be used for the electrochemical detection of dopamine and ephedrine. BLMs were composed of egg phosphatidylcholine and 35% (w/w) dipalmitoyl phosphatidic acid in which the receptor was incorporated. These BLMs modified with the resorcin[4]arene receptor can be used as one-shot sensors for the direct electrochemical sensing of these energizing–stimulating substances. The interactions of these compounds with the lipid membranes were found to be electrochemically transduced in the form of a transient current signal with a duration of seconds, which reproducibly appeared within 8 and 20 s after exposure of the membranes to dopamine and ephedrine, respectively. The response time for BLMs without the receptor for dopamine was about 3 min, whereas no signals were obtained for ephedrine in the absence of the receptor. The mechanism of signal generation was investigated by differential scanning calorimetric studies. These studies revealed that the adsorption of the receptor is through the hydrophobic tails of the receptor, whereas hydrophilic groups of the receptor were directed towards the electrolyte solution enhancing the ion transport through the lipid membranes. The magnitude of the transient current signal was related to the concentration of the stimulating agent in bulk solution in the micromolar range. No interferences from ascorbic acid were noticed because of the use of the negatively charged lipids in membranes. The present technique can be used as one-shot sensor for the detection of these pharmaceutical substances and future research is targeted to the determination of these chemicals in human biofluids such as urine of athletes. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Biosensor; Electrochemistry; Differential scanning calorimetry; Bilayer lipid membrane; Resorcin[4]arene receptor; Dopamine; Ephedrine

## 1. Introduction

Dopamine and ephedrine are widely used as energizing–stimulating substances and for exciting central nervous system in various diet foods and athletes.

Many pharmaceutical companies use this class of stimulants alone or in combination. These catecholamines are sympathetic neurotransmitters and play an important role in the metabolism of sugars, lipids, etc. [1]. Ephedrine is also used for curing of bronchial asthma [2]. The extensive use of these chemicals concerns guidelines of international athlete associations and has generated significant interest for development of methods for the rapid detection of these

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compounds in health foods, pharmaceutical products and human fluids of athletes. High-performance liquid chromatography methods have widely been used for the determination of these compounds [3,4]. These methods however are often complicated and expensive. Electrochemical sensors using modified electrodes have recently been used for the rapid detection of these stimulants [5,6].

Lipid films have been widely used for the rapid detection or continuous monitoring of a wide range of compounds in foods and in the environment [7]. Such electrochemical detectors are simple to fabricate and can provide fast response and high sensitivity. However, in many cases the use of a receptor is required. This work explores the incorporation of a novel calix-4-resorcinarene receptor in planar freely suspended bilayer lipid membranes (BLMs) for the selective detection of dopamine and ephedrine. This class of compounds has recently appeared in the literature [8] and they were widely used as receptors in biosensors for the detection of metals [9–11]. Recent work has investigated the selectivity of these resorcin[4]arene receptors towards catecholamines [12]. BLMs were composed of egg phosphatidylcholine (PC) and 35% (w/w) dipalmitoyl phosphatidic acid (DPPA). Such studies of interactions of dopamine and ephedrine with lipid membranes have not been reported in the literature up to date. The interactions of the stimulating substances with these BLMs produced transient electrochemical current signals with a duration of seconds, which reproducibly appeared within 8 and 20 s after exposure of the membranes to dopamine and ephedrine, respectively. The magnitude of this signal could be used to quantify the concentration of the stimulant. The response time for BLMs without the receptor for dopamine was about 3 min, whereas no signals were obtained for ephedrine in the absence of the receptor. Determination of the mechanism of signal generation involved analysis of the structural effects caused by interactions of the stimulants with model lipid membranes. Differential scanning calorimetry (DSC) studies have shown that the resorcin[4]arene receptor is embodied in the lipid film through the hydrophobic tails of the receptor, whereas the hydrophilic groups of the receptor were directed towards the electrolyte solution enhancing the ion preconcentration at the surface of the lipid membranes. The DSC studies also have

shown that the interactions of ephedrine and dopamine with lipid vesicles with the incorporated receptor alter the phase structure of the lipid films to a more liquid crystalline phase that facilitates ion transport through the lipid membrane.

## 2. Materials and methods

### 2.1. Chemicals

Lyophilized egg PC and DPPA were supplied by Sigma, St. Louis, MO, USA, and were used as lipids for the formation of BLMs and monolayer membranes. Dopamine was also purchased from Sigma. Ephedrine hydrochloride was supplied from Aldrich (Aldrich-Chemie, Steinheim, Germany). Water was purified by passage through a Milli-Q cartridge filtering system (Milli-Q, Millipore, El Paso, TX, USA) and had minimum resistivity of 18 M $\Omega$  cm. All other chemicals were of analytical reagent grade.

The equipment for the preparation of 'solventless' or 'solvent-free' BLMs has been described in detail elsewhere [13]. These membranes were formed in a circular orifice of 0.32 mm diameter that was located in a Saran Wrap (PVDC; DowBrands, L.P., Indianapolis, IN, USA) partition of a thickness of ca. 10  $\mu$ m. The plastic partition was used to separate two identical solution chambers made from polymethylmethacrylate. Each solution compartment had a volume of ca. 10 cm<sup>3</sup> and an air/electrolyte interface area of 3 cm<sup>2</sup>. An external voltage of 25 mV d.c. was applied across the BLMs between two Ag/AgCl reference electrodes. A digital electrometer (Model 614, Keithley Instruments, Cleveland, OH, USA) was used as current-to-voltage converter. The electrochemical cell and the electronic equipment were isolated in a grounded Faraday cage.

A Perkin-Elmer differential scanning calorimeter (Model DSC-7) was used for the DSC experiments; the thermograms were processed by means of the Thermal Analysis Data Station of the DSC-7.

### 2.2. Measurements

The dilute lipid solutions used for the formation of solventless freely suspended BLMs were composed of 35% (w/w) DPPA and contained 0.2 mg ml<sup>-1</sup> total

lipid. This solution contained  $0.13 \text{ mg ml}^{-1}$  receptor. Lipid solutions were prepared daily from a stock solution of PC and DPPA (each  $2.5 \text{ mg ml}^{-1}$ ) in *n*-hexane and absolute ethanol (80+20). The stock solution of the receptor contained  $2.4 \text{ mg ml}^{-1}$  and was stable for about 1 week. The stock lipid and receptor solutions were stored in darkness in a nitrogen atmosphere at  $-4^\circ\text{C}$ . The BLMs were formed in a  $0.1 \text{ M}$  KCl electrolyte solution (pH adjusted with  $50 \text{ mM}$  HEPES at 7.0).

Solventless BLMs were used in these studies to avoid any structural effects caused by the presence of residual solvent, and were prepared as previously described [13]. A volume of ca.  $10 \text{ }\mu\text{l}$  of dilute lipid solution containing the receptor was added drop-wise from a  $\mu\text{l}$  syringe to the water surface in one cell compartment near the partition. The accompanying solvent (*n*-hexane) was allowed sufficient time (ca. 5 min) to completely evaporate before the process of monolayer casting. Over a period of a few seconds the electrolyte level in one solution compartment was brought below the orifice and was then raised again with a  $10 \text{ ml}$  disposable syringe that was extended with plastic tubing. The formation of a membrane was verified by the magnitude of the ion current, and the electrical properties of the membranes [13]. When the ion current stabilized (over a period of about 5 min), the solution of dopamine or ephedrine was injected in one solution compartment while continuous stirring. All electrochemical experiments were done at  $25 \pm 1^\circ\text{C}$ .

Lipid vesicles composed of PC and 35% (w/w) DPPA ( $2.5 \text{ mg ml}^{-1}$  of total lipid) were used for the DSC experiments [14–16]. These vesicles were prepared in *n*-hexane. The organic solvent of the lipid solution was evaporated under a stream of nitrogen gas. The lipid was resuspended by vortexing in a  $0.1 \text{ M}$  KCl aqueous solution, at a temperature above the estimated phase transition temperature,  $T_m$ , of the vesicles (i.e.  $38.3^\circ\text{C}$  in our experiments). The suspensions had a concentration of  $5.0 \text{ mg ml}^{-1}$  total lipid and were left refrigerated overnight. An amount of  $20 \text{ }\mu\text{l}$  of lipid suspension without or with the receptor ( $0.26 \text{ mg ml}^{-1}$ ) was withdrawn using a calibrated microsyringe and mixed into an aluminum pan with  $20 \text{ }\mu\text{l}$  of HEPES solution ( $0.1 \text{ M}$ , pH 7.0). The experiments were repeated with these compounds using  $20 \text{ }\mu\text{l}$  of either identically buffered do-

pamine solution ( $2.0$  or  $8.0$  or  $13 \text{ }\mu\text{M}$ ) or ephedrine ( $0.120$  or  $1.2$  or  $2.13 \text{ mM}$ ) instead of the HEPES solution. The pan was then hermetically sealed. The vesicle suspensions were scanned between  $25$  and  $40^\circ\text{C}$  with a scanning rate of  $2^\circ\text{C min}^{-1}$  (buffer solution was the control). Initiation of scanning was done 5 min after mixing of the lipid suspension with dopamine or ephedrine solution to ensure equilibration of adsorption of these compounds onto BLMs.

### 3. Results and discussion

#### 3.1. Electrochemical investigation of interactions of BLMs containing the receptor with dopamine and ephedrine

Fig. 1A shows recordings of the signals obtained from BLMs composed of PC and 35% (w/w) DPPA and containing the receptor for different concentra-

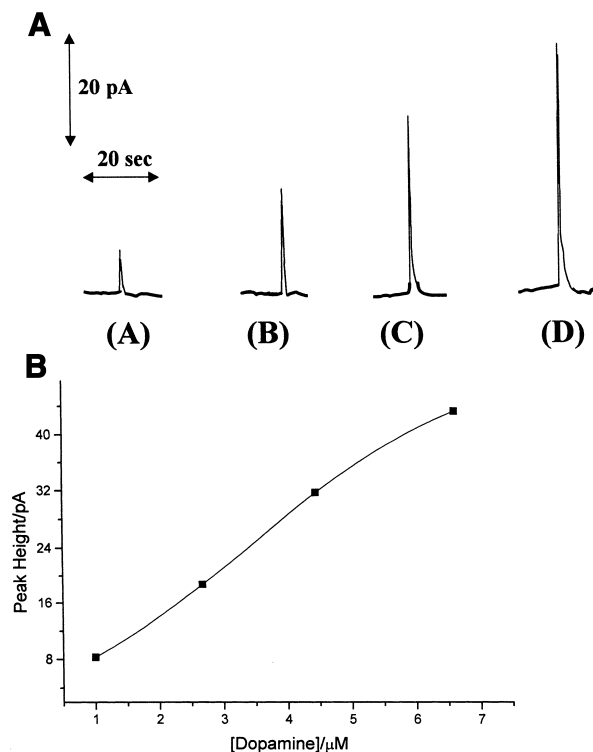


Fig. 1. (A) Recordings obtained with BLMs composed of PC and the following dopamine concentrations in bulk electrolyte solution ( $\mu\text{M}$ ): (A) 1.00; (B) 2.67; (C) 4.44; (D) 6.50. The injection of each sample was made at the beginning of each recording. (B) Calibration of results of A.

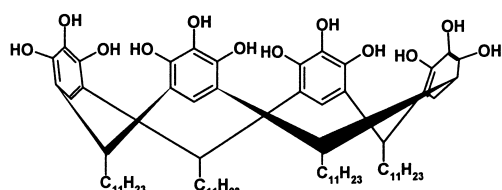


Fig. 2. Chemical structure of resorcin[4]arene receptor.

tions of dopamine. It can be seen that a transient ion current response appears at a relatively constant time after exposure of the membrane to dopamine ( $8 \pm 1.2$  s,  $n = 5$ ). This time is longer than the mixing time of 3 s (as observed with the use of methyl orange as an indicator). These transient responses appeared as singular events (no further transients were observed over a period of 10 min). The results indicate that the reproducibility of the time of appearance of the signal is on the order of ca.  $\pm 15\%$  and the magnitude of the transient of current is increased with an increase of the concentration of dopamine. The magnitude of the transient signals can be used to quantify the concentration of dopamine between 0.65 and 6.5  $\mu\text{M}$ . The heights of the transients may be linearly related to the concentration of dopamine in bulk solution with the regression equation:  $\Delta I$  (pA) =  $6.22 C$  ( $\mu\text{M}$ ) + 2.19,  $r^2 = 0.994$ . The blank value (i.e. the amplitude of the transient for zero concentrations of dopamine) of 2.19 is due to the fitting procedure. The equation that relates the magnitude of heights of the transients versus concentration of dopamine can also be described using a Boltzmann sigmoidal (see Fig. 1B):

$$\Delta I \text{ (pA)} = 51.2 - [54.4 / 1 + e^{(-3.38 + x/1.81)}] \quad (r^2 = 0.9998) \quad (1)$$

where  $x$  represents the concentration of dopamine in  $\mu\text{M}$ . The detection limit (i.e. the lowest concentration that could be measured) was 0.5  $\mu\text{M}$ . The reproducibility of chemical sensing of dopamine by the use of the height of transients is between  $\pm 3\%$  and  $\pm 8\%$  (e.g. RSD = 6.2% for 4.44  $\mu\text{M}$  of dopamine,  $n = 5$ ). Dopamine additions have been made at different times after membrane formation and stabilization. Only after addition of dopamine were the transient currents of Fig. 1A observed. The chemical structure of the receptor is shown in Fig. 2. BLMs without receptor have provided similar transients, but these

transients appeared about 3 min after addition of dopamine. The results indicated that the analytically useful concentration range for dopamine determination is also between 0.65 and 6.5  $\mu\text{M}$  and the current was also related to dopamine concentration ( $\Delta I$  (pA) =  $4.1 C$  ( $\mu\text{M}$ ) - 0.58,  $r^2 = 0.995$ ). The detection limit (based on the lowest concentration that could be measured) was 0.6  $\mu\text{M}$ . Similar results were obtained with ephedrine. The relationship between signal magnitude and ephedrine concentration fits a linear relationship ( $\Delta I$  (pA) =  $0.161 C$  ( $\mu\text{M}$ ) - 0.30,  $r^2 = 0.9992$ ). The results have indicated that the analytically useful concentration range for ephedrine determination is between 50 and 500  $\mu\text{M}$ . For higher concentrations of the stimulants, the graphs decline from linearity probably due to the saturation of the lipid membrane with the stimulant. The detection limit for ephedrine for S/N = 3 was 25  $\mu\text{M}$ .

The transient signals obtained by stimulant/lipid membrane interactions are intriguing due to the reproducibility and speed of signal development at even low concentrations of the stimulants. The transient currents have a duration of seconds and are indicative of reorganization of charge at the surface of membranes. No reports on the interactions of a stimulant with lipid membranes have been reported in the literature. The stimulants used in these studies are polar or charged at the pH used (Fig. 3) [17]. The pH effect on the signal magnitude of BLMs formed from PC and 35% (w/w) DPPA was examined in the pH range of 4.0–8.0 [18]. These results have shown that the ion current through such BLMs did not depend on the pH value between pH values 6 and 7. PC is an amphiphilic molecule above pH 3.0 [18], as would be the case in the present work where so-

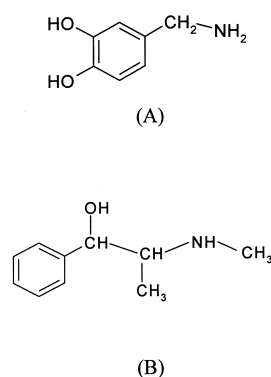


Fig. 3. Chemical structure of (A) dopamine and (B) ephedrine.

lutions were buffered to pH 7.0. Ephedrine is a weak base [17] and has a  $pK_a$  value of pH 4.6. It is likely that the cation of ephedrine interacts with the negatively DPPA molecules at the BLM surface through electrostatic forces and pre-concentrates at the lipid membrane surface. Dopamine shows similar interactions.

The data in Fig. 1A indicate that for all stimulant concentrations tested, it takes about the same delay time for the signal to be observed. The relatively invariable delay time does not appear to be related to the stimulant concentration, and suggests a mechanism of response that may occur in two steps: stimulant adsorption to BLM surface, which may then associate to provide electrostatic perturbation of the lipid membrane. This second association step seems relatively independent of the rate of the first step (adsorption), the former being the rate determining step. At any one temperature (our data have only been collected at room temperature, *vide supra*) the kinetics process of association should be relatively constant, and is expected to be slow as based on movement across the surface of membranes. At higher concentrations of the stimulant, the association effects may be larger in size or number and cause a greater effect; therefore the signal would be related to the stimulant concentration.

### 3.2. DSC investigations

To further investigate the mechanism of signal transduction, DSC experiments using vesicular BLMs without and with the receptor molecules in the presence of dopamine and ephedrine were done. The transition temperature of lipid vesicles composed of PC and 35% (w/w) DPPA with incorporated receptor was investigated in the presence of these stimulants. Vesicles composed of PC and 35% (w/w) DPPA without incorporated receptor have provided a pre-transition temperature of 29.8°C, whereas the main transition temperature of these vesicles was 36.2°C (Fig. 4A). The pre-transition temperature of vesicles with incorporated receptor was practically constant, whereas the transition temperature was increased to 37.5°C (Fig. 4B). The  $\Delta H$  of the pre-transition and main transition temperatures without the receptor was 0.38 and 0.13 cal g<sup>-1</sup>. In the presence of the receptor molecules these values were 0.19 and

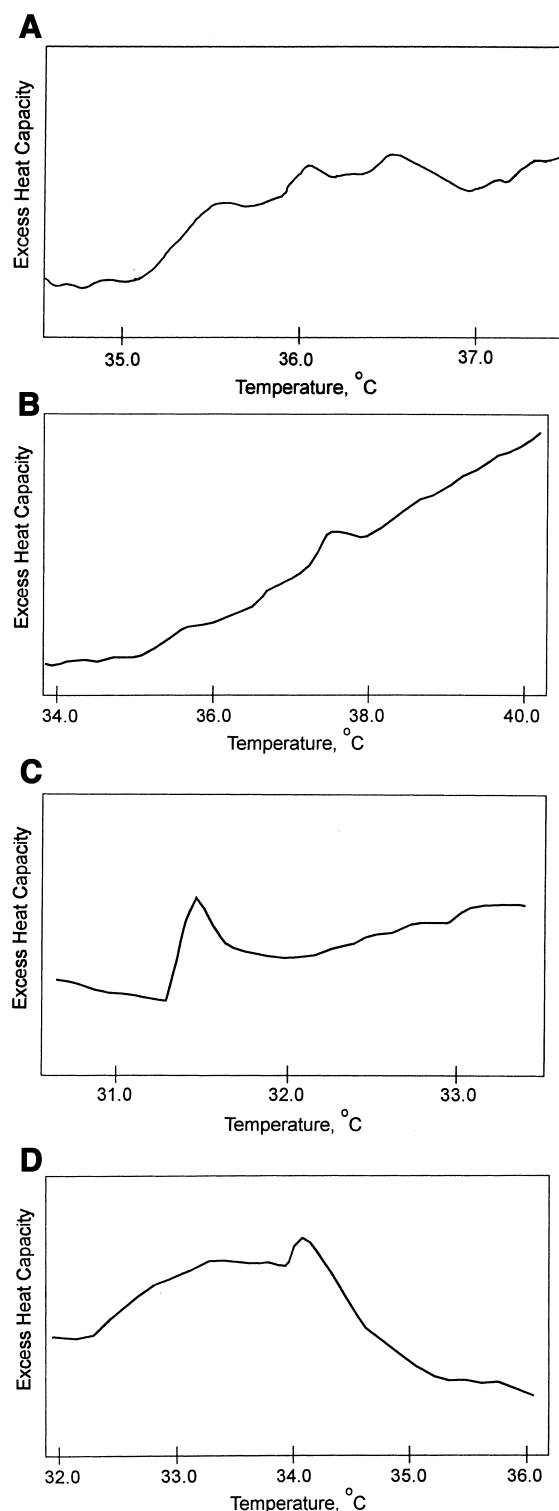


Fig. 4. DSC thermograms of vesicles composed of 35% DPPA at pH value 7.0: (A) in the absence of calix-4-resorcinarene; (B) in the presence of the receptor; (C) 8  $\mu$ M dopamine and (D) 2.13 mM ephedrine.

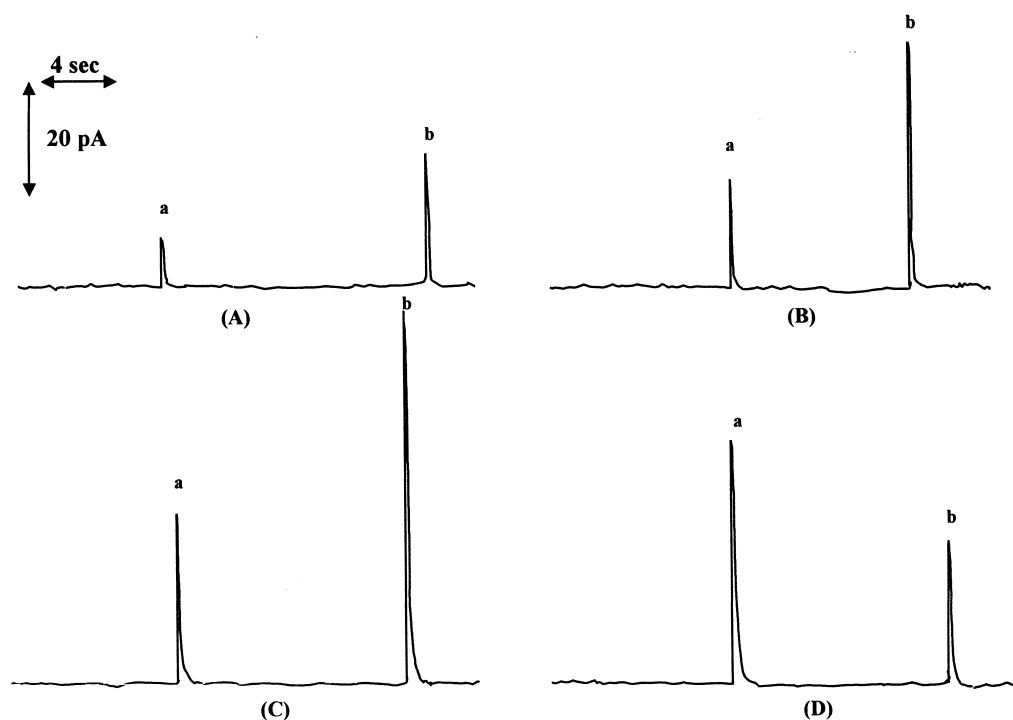


Fig. 5. Experimental results obtained for mixtures of dopamine and ephedrine with BLMs. The solutions injected contained: (A) dopamine 1.00  $\mu\text{M}$  and ephedrine 147  $\mu\text{M}$ . (B) Dopamine 2.67  $\mu\text{M}$  and ephedrine 267  $\mu\text{M}$ . (C) Dopamine 4.44  $\mu\text{M}$  and ephedrine 400  $\mu\text{M}$ . (D) Dopamine 6.67  $\mu\text{M}$  and ephedrine 147  $\mu\text{M}$ . The injection of each sample was made at the beginning of each recording.

$0.05 \text{ cal g}^{-1}$ . In the presence of dopamine and ephedrine, the pre-transition temperature remained constant and the  $\Delta H$  values remain constant. The main transition temperature was decreased, for example, to 31.5 and 34.1°C for 8  $\mu\text{M}$  dopamine and 2.13 mM ephedrine, respectively (Fig. 4C,D). These results show that the receptor increases the transition temperature of lipid vesicles and is therefore incorporated in the lipid membrane through its hydrophobic tails leaving its hydrophilic groups towards the electrolyte solution. The results show that the incorporation of the receptor in the lipid membrane stabilizes the gel phase of the lipid film. The incorporation of the alkyl chains of the receptor results in the appearance of greater order in the bilayers that is reflected by the increased  $T_m$  value of the liposomes. In the presence of dopamine or ephedrine, the gel phase of the lipid membranes is destabilized which is reflected by the decrease of the  $T_m$  value of the liposomes [19,20]. The fluidity of these vesicles in the presence of these substances is increased which is in agreement with the electrochemical signals. These results are in agreement with the mechanism that was

previously described and is based on both electrochemical double layer and cation concentration changes at the lipid/water interface [15,18,21,22–24].

### 3.3. Use of interactions of BLMs with stimulants for biosensors: analysis of mixtures of dopamine and ephedrine

The magnitude and sensitivity of the signal for the stimulant determination depend on the surface charge of the lipid membrane. The introduction of a negatively charged lipid such as DPPA to BLMs composed of PC can substantially alter the fluidity and/or electrostatic fields of BLMs [18,24]. The sensitivity of the determination increased with an increase of DPPA concentration. This is a direct result from the fact that DPPA is negatively charged at the pH used, and the stimulant is preconcentrated at the lipid membrane surface.

There is significant variability of peak area and shape for the ion current transients. The variability of peak area suggests that peak height is a better indicator in the determination of dopamine and

ephedrine. The duration (relaxation time, etc.) of the transient response versus analyte type was found not to depend on the compound and whether this was dopamine or ephedrine. The time period of transient peaks was always in the range of approximately 1–2.5 s. The evolution of the transient signal occurs because of dynamic changes at the membrane–solution interface and is not directly influenced by the presence of dopamine or ephedrine in solution. However, the time delay for the appearance of these transients was different for dopamine and ephedrine. The range of time delay for the appearance of these transients was between 7 and 11 s for dopamine and 19–23 s for ephedrine ( $n = 15$ ).

The difference observed in the time for the appearance of signal for dopamine and ephedrine has allowed the investigation of analysis of a mixture of these two compounds. Fig. 5 shows recordings obtained for such mixtures containing variable amounts of these stimulants. This figure shows that a discrete signal is obtained for each compound in mixture. The resolution of the peaks of each stimulant obtained in such mixtures was sufficient, to permit reliable selective monitoring of these in mixture. The results shown in Fig. 5 were used for quantification of each compound in mixtures and the results are summarized in Table 1. This table shows that the recovery of the stimulant in mixture was complete.

Interference studies were done with the present sensor (in a competitive study, i.e. both stimulant and interferent together in solution) and included investigation of most commonly found compounds in pharmaceutical preparations such as ascorbic

acid (100+1). No significant interferences were noticed from the presence of this compound at the ratios that were examined and the error was less than  $\pm 5\%$ . For example, the relative error was 0.7% and 4.9% for mixtures of ascorbic acid with dopamine at a ratio of about 2+1 and 75+1, respectively. Note that real samples of pharmaceutical preparations mainly contain ascorbic acid and dopamine. There are, however, other real samples such as urine of athletes that contain other constituents, i.e. proteins. The disadvantage of the present sensor as compared with conventional analytical methods [3,4] is the fragility and the very limited stability of the freely suspended BLMs. Also, the sensitivity of these techniques is better than the present method; for example, the limit of quantification (LOQ) in [3] is about 10  $\mu\text{M}$  of ephedrine, whereas in [4] the LOQ was about 20  $\text{ng ml}^{-1}$  for catecholamines (which corresponds to about 0.1  $\mu\text{M}$  for dopamine). The concentration of catecholamines in human plasma and urine varies between 0.05 and 12  $\mu\text{M}$  [3,4]. The concentration of ephedrine in human plasma samples of athletes that use this stimulant was found above 10  $\mu\text{M}$  [3]. This concentration is below the limit of detection of the present method. However, the present technique can be used as one-shot sensor for the rapid detection of these stimulants. The method could also be potentially applied for the selective determination and analysis of mixtures of dopamine and ephedrine in urine of athletes by using filter-supported BLMs [14]. Research is therefore targeted to the application of the present technique in real samples containing these stimulants that may also contain proteins, such as urine of athletes.

In conclusion, the present article investigates an interaction between an amphiphilic molecule such as dopamine and ephedrine with lipid bilayer. Similar investigations of other amphiphilic molecules with lipid membranes have recently been published [25]. These later studies include the interactions of lipid bilayers with quercetin which is a strong antioxidant bioflavonoid product synthesized in many green and higher plants and may be a potential anti-cancer agent that promotes apoptosis of tumor cells. This variety of studies of interactions between amphiphilic molecules and artificial lipid membranes promotes our knowledge about interactions of such molecules with living cells.

Table 1

Results of quantification of mixtures of dopamine and ephedrine (the numbers in parentheses are the taken amounts of stimulants)

Sample no.	Dopamine ( $\mu\text{M}$ )	Ephedrine ( $\mu\text{M}$ )
1	1.05 (1.00)	144 (147)
2	2.63 (2.67)	265 (267)
3	4.40 (4.44)	405 (400)
4	0.980 (1.00)	270 (267)
5	0.820 (0.778)	395 (400)
6	2.71 (2.67)	61.0 (60.0)
7	2.60 (2.67)	328 (333)
8	6.45 (6.67)	152 (147)
9	4.50 (4.44)	143 (147)
10	6.69 (6.67)	61.2 (60.0)

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